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(54) PHARMACEUTICAL COMPOSITIONS CONTAINING BISDITHIOHETEROCYCLIC REAGENTS

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ABSTRACT

This invention relates to a method of modifying intercellular reactions in mammals by administering a relatively non-toxic reagent which preferentially reacts with peripheral sulfhydryl groups present about mammalian cells rather than those sulfhydryl groups which are present in the cell. The reagent employed is a dithio compound having the structure R-S-S-R typically 6,6'-dithiodinicotinic acid or the sodium salt thereof. This invention also relates to compositions containing the active reagents.

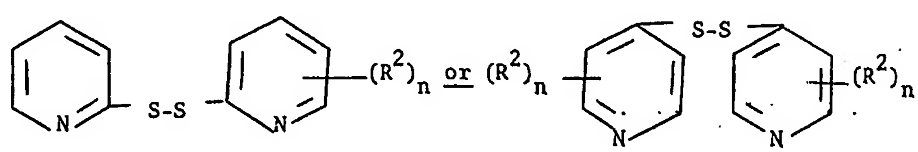
THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A composition for blocking tumor metastasis and invasiveness, containing as the active ingredient a substance of the formula

R-S-S-R

wherein the Rs represent organic radicals together with pharmaceutically acceptable diluents.

2. A composition as in claim 1, wherein the active ingredient has the structure



wherein n is an integer having a value of 1 to 4 and the R^2s represent carboxy groups or salts or other derivatives of said carboxy groups.

3. A composition as in claim 1, wherein the active ingredient is 6,6'-dithiodinicotinic acid.

4. A pharmaceutical composition which comprises a reagent as defined in claim 2 in association with a pharmaceutical acceptable medium suitable for intravenous or intramuscular or a pharmaceutically acceptable carrier in a form suitable for oral administration.

5. A composition according to claim 1 for modification of intercellular reactions of mammalian cells, consisting essentially of a non-toxic dithiobisheterocyclic compound which (a) forms a substantially smaller quantity of thione product by reaction with whole cells than by reaction with a homogenate of the same cells and which (b) does not alter the rates of cell respiration and glycolysis, dispersed in a physiologically acceptable buffer solution.

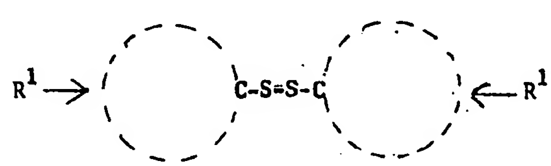
6. A composition according to claim 1 or 5 in which the active ingredient is 6,6'-dithiodinicotinic acid.

It has been discovered that cellular surface reactions can be modified by bringing the cell into reactive engagement with a reagent which is capable of blocking the sulfhydryl groups of the cell surface.

It has been discovered, more particularly, that the processes of tumor metastasis and invasiveness in living mammals can be inhibited by administering to said mammals a non-toxic reagent which is capable of preferentially reacting with peripheral sulfhydryl groups present about mammalian cells rather than with those sulfhydryl groups which are present within the cell. Preferably, the reagent employed is one which, in addition to blocking the peripheral sulfhydryl groups of the cells, also has the effect of modifying the electric charge of the cell surface.

It has also been discovered that various types of dithio compounds, all having the structure R-S-S-R, where each represents an organic radical, have the ability to react preferentially with the peripheral sulfhydryl groups present about the cell. Further, those of said dithio compounds wherein the R radicals incorporate groups (such, for example, as -COOH or $-SO_3H$) which have a negative charge under pH conditions above about 7, have the ability to modify the electric charge of the cell surface.

Of the compounds which have been found to react preferably with the peripheral sulfhydryl groups of the cell, those such as N,N'-diacetylcystamine and beta-mercaptopropionic acid disulfide are of the linear type. Many of the others, however, fall into a class referred to herein as bisdithioheterocyclic compounds. The latter compounds, or reagents, have the structure:



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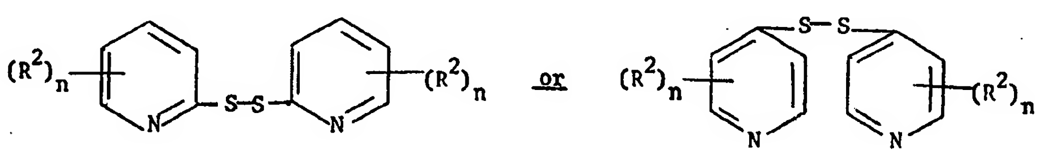
wherein the R^1s, which may be the same as or different from one another, represent heterocyclic radicals containing from 1 to 5 ring nitrogen atoms and optionally sulfur in the ring, along with carbon. Said radicals may be substituted or unsubstituted, and can represent single or fused aromatic rings.

It has been discovered that within this broad class of bisdithioheterocyclic compounds there exist various compounds and groups of compounds which have the capacity to react preferentially with peripheral cellular sulfhydryl groups rather than with those which are present within the cell. One such group of compounds has been found to be that wherein the R^1s represent pyridyl nuclei substituted by anionic groups, said compounds having unusual characteristics which make them particularly well adapted to be employed in mammalian systems for the reversible blocking of peripheral cellular sulfhydryl groups. More specifically, said compounds appear to be essentially non-toxic while also having the ability to react preferentially with peripheral rather than with intracellular sulfhydryl groups. Further, said compounds have the desired ability to modify the electric charge of the treated cell surface. The compounds in this preferred group have one or the other of the structures:



(II) or (III)

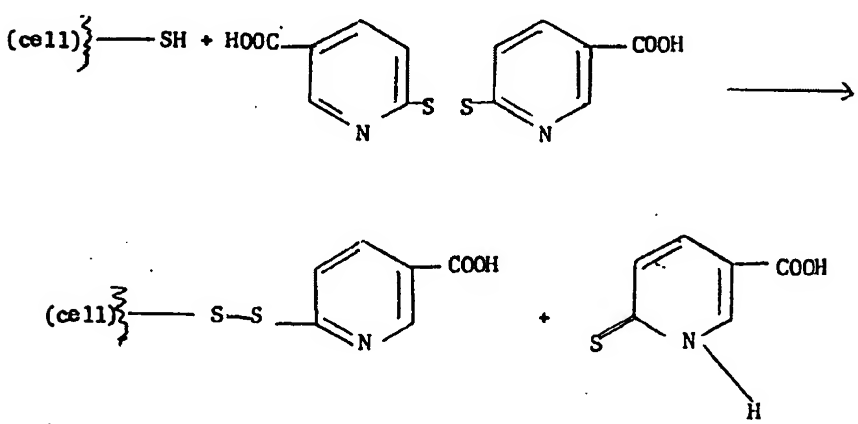
wherein n represents a whole integer having a value of from 1 to 4, and the R^2s represent carboxy groups or the salt, ester or amide derivatives of said carboxy groups. For example, suitable salts are, for example, those formed with alkali or alkaline earth metals, with ammonia or with amines such as cyclohexylamine, morpholine or other aliphatic, alicyclic, aromatic or heterocyclic amines. Representative ester moities are those such as methyl, ethyl and higher alkyl groups, as well as cyclohexyl and other alicyclic groups. Representative amide groups for substitution on the pyridyl nuclei include $-CONH_2$, as well as those wherein one or both hydrogen atoms on the amide nitrogen atom are replaced by aliphatic, heterocyclic, alicyclic or aromatic groups, including those of a substituted character; typical groups are beta-aminoethanol and morpholine.

Representative compounds coming within the group represented by structures (II) or (III) above include: 4,4'-dithiodinicotinic acid; 6,6'-dithiodinicotinic acid, 2,2'-dithiodinicotinic acid, 2,2'-dithiobis-(isonicotinic

acid), 6.6'-dithiodipicolinic acid, 4,4'-dithiodipicolinic acid, 4,4'-dithiodinicotinic acid sodium salt, 6,6'-dithiodinicotinic acid sodium salt, 2,2'-dithiodinicotinic acid sodium salt, 6,6'-dithiodipicolinic acid sodium salt, 2,2'-dithiobis-(isonicotinic acid)-sodium salt, 6,6'-dithiodinicotinic acid potassium salt, 6,6'-dithiodinicotinic acid magnesium salt, 6,6'-dithiodinicotinic acid ammonium salt, 4,4'-dithiodipicolinic acid potassium salt, 4,4'-dithiobis(2,5-pyridinedicarboxylic acid), 4,4'-dithiobis-(2,6-pyridinedicarboxylic acid), 2,2'-dithiobis-(3,4-pyridinedicarboxylic acid),4,4'-dithiobis-(3,5-pyridinedicarboxylic acid), 4,4'-dithiobis-(2,6-pyridinedicarboxylic acid sodium salt), 4,4'-dithiobis-(2,3,5-pyridinetricarboxylic acid), 2,2'-dithiobis-(3,4,5-pyridinetricarboxylic acid) and 2,2'-dithiobis-(3,4,5,6-pyridinetetracarboxylic acid).

Good results can also be obtained with compounds having the structure of II or III above, except that the pyridyl rings are substituted by $-CH_3$ or other alkyls, -OH, -CN, halogen or -CNO. The -COOH or derivative groups thereof can also be present on the ring, if desired.

All the forgoing compounds corresponding to the II or III structure are capable of reacting with a sulfhydryl group to form a thione product, said compounds thus being referred to as "thione-forming" reagents. A typical reaction which takes place between a compound of this class (6,6'-dithiodinicotinic acid) and a peripheral -SH group on a cell can be represented as follows, where the reaction product not linked to the cell is 6-thiononicotinic acid:



(2)

In addition to the exemplary 6,6'-dithiodinicotinic acid reagent referred to above, other representative dithiobisheterocyclic reagents which

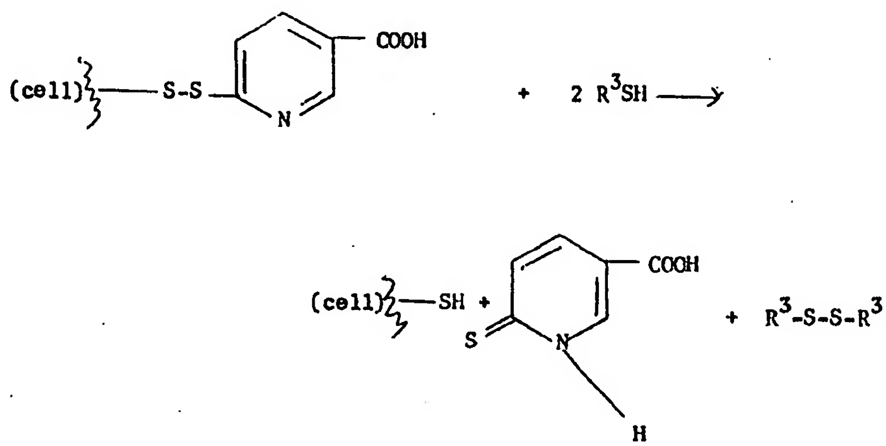
have been found to be capable of preferentially reacting with peripheral sulfhydryl groups of cells in the above fashion include 2-mercaptouracil disulfide 6,6-dithiodiisonicotinic acid and 2,2'-dithiobis-(5-amidopyridine), together with the sodium salts of said compounds which incorporate carboxyl groups. The latter groups, whether present as such or in salt form, bear a negative charge when at a pH of at least 7 and thus are capable of modifying the electric charge of the cell which has been treated with these reagents.

It is also possible to effect the desired blockage of peripheral cellular sulfhydryl groups by using bisdithioheterocyclic reagent compounds wherein the substituted pyridyl nuclei, as referred to above in connection with structures II and III, are linked by -S-S- in the 3- position of the said nuclei. Here, however, the reaction may not be formative of a thione type of reaction byproduct.

Compounds of this type which bear anionic substituent groups also have the ability to modify the surface charge on the cell. It will be noted that a linear disulfide such as the beta-mercaptopropionic acid disulfide mentioned above, while not a thione-forming reagent, is also capable of modifying the cellular charge while reacting preferentially with the peripheral -SH groups on the cell.

It has also been discovered that when treating mammalian cells with a bisdithioheterocyclic or other organic disulfide reagent capable of reacting in this preferential fashion, it is possible to reversibly block all, or essentially all, of the peripheral cellular sulfhydryl groups without altering the rate of cellular respiration and glycolysis to any significant extent. Moreover, the blocking reaction is a reversible one. Thus, when said reaction occurs in the body of a living mammal the blocked groups return to their original -SH form within a relatively short period of time (usually several hours) as a result of conventional metabolic processes, assuming no further amounts of the cyclic reagent are available for reaction in the meantime. The administration of a thiol of the type found in mammalian systems such, for example, as glutathione, will also induce restoration of free peripheral -SH groups at a rapid rate. This deblocking reaction, wherein glutathione is

indicated by R^3SH, can be illustrated as follows using 6,6'-dithiodinicotinic acid as the reagent:



Repeated or continuous administration of the dithiobisheterocyclic or other organic disulfide reagent to the mammal will, of course, maintain the cellular system in any desired condition of peripheral sulfhydryl group blocking without damage to the cell.

For convenience of description, compounds corresponding to structures (II) or (III), as well as those wherein similarly substituted pyridyl nuclei are disulfide linked through the 3-position, are collectively referred to herein and in the claims as "dithiobis-(carboxypyridine)" compounds, or more simply as "dicyclic" reagents. Said compounds can readily be prepared by the oxidation of the corresponding mononuclear precursor compounds wherein the pyridyl nucleus, in addition to the carboxy or carboxy derivative group(s), or other substituent group, also carries a sulfhydryl group (-SH) attached to a ring carbon atom. In the ensuing oxidation reaction, two molecules of the starting compound link up through the sulfur atoms of the sulfhydryl groups. This oxidation can readily be carried out by using hydrogen peroxide as employed in the stoichiometrically required amount or in slight excess. The reaction is conducted at temperatures below about 35°C. in either an aqueous medium or in a solvent such as benzene or acetone in which the precursor compound is soluble. Alternatively, the oxidation can be effected by the addition of iodine to a solution of the said thiol precursor in an appropriate aqueous or other solvent medium in the presence of sodium iodide or potassium iodide. The iodine can be

added either per se or, for example, in the form of a solution in aqueous potassium iodide, the reaction being considered complete when the iodine color persists in the stirred solution. The desired product compound separates out as a precipitate and can be recovered and purified by conventional methods, the usual practice being to filter off the compound and then to wash it with water and/or acetone. In order to determine which reagents are capable of preferentially reacting with peripheral cellular groups, an excess of the reagent can be reacted first with a mass of intact cells and then with a like mass of homogenized, or comminuted cells of the same kind. When the relative amount of the product compound (e.g. the thione product) which is formed by reaction with whole cells is very substantially lower than the amount formed during reaction with the homogenate, the desired preferential attribute is present. This method will be described below in Example 1.

Example 1

Ehrlich ascites tumor cells were harvested from 7- to 10-day transplants in female Swiss mice and then washed free of blood in the manner described below in Example 2. One portion of the cells was then left as such, while the other was converted to a homogenate in which essentially no whole cells were left. 15 mg. samples (dry weight) of the intact and of the homogenized cells were incubated for 1 hour at 37°C. with 5 ml. of KRP buffer containing 5 μmoles of one or another of the three disulfide reagents enumerated below in Table 2 with shakin in air. This amount was sufficient to provide a system containing the reagents in a concentration of 1×10^{-3}M. After filtration through glass paper (Whatman GF/C), the amount of thione produced was determined from the absorbance at the wavelength indicated in the table. In the case of 2,2'-dithiodipyridine reactant, the amount of thione product formed by reaction with whole cells was at least as high (9.61 μ moles) as that formed from the homogenate (6.52 μ moles), the higher value obtained in the case of intact cells being attributable to enzymatic action occurring in intact or metabolically active cells. Each of these values was then taken as 100% in Table 1 below. The other two reagents gave approximately this same amount of thione product when reacted with the homogenate; however, the amount of thione provided by these two reagents when reacted with whole cells was but 20 percent

of the value obtained with the 2,2'-dithiodipyridine reactant. This indicates that these dithiobis-(carboxypyridine) reagents are selectively coupling themselves to peripheral rather than to intracellular sulfhydryl groups. These data are expressed below in Table 1.

Table 1

EXTENT OF REACTION OF DITHIOBISHETEROCYCLIC COMPOUNDS WITH EHRLICH ASCITES CELLS AND HOMOGENATES

Reagent	pH	Characteristic Thione λ_{max} (mu)	Extent of Reaction	
			Intact Cells	Homogenate
2,2'-dithiodipyridine	7.2 or 8.0	343	100%	100%
6,6'-dithiodinicotinic acid	7.2	344	20	102
2,2'-dithiobis-(isonicotinic acid)	7.2	365	20	94

In companion operations conducted in essentially the same fashion but using the corresponding sodium salts of 6,6-dithiodinicotinic acid and of 2,2'-dithiobis-(isonicotinic acid) rather than the free acids, the thione product values obtained are essentially the same as those reported in Table 1 above.

Example 2

Ehrlich ascites fluid was harvested from 7- to 10-day transplants in female Swiss mice; the cells were separated by centrifugation at low speed, then suspended in KRP buffer (Krebs-Ringer phosphate buffer at pH 7.2 ± 0.2) and centrifuged again. The cells were then washed with KRP buffer for several times in the presence of heparin (20 U.S.P units/ml.) to remove red blood cells. The cells were resuspended in the KRP buffer to yield 800 x 10^6 cells in 10 ml. of buffer and then incubated for 5 minutes at 25°C. in the absence (control) and presence of 10 μmoles of 6,6'-dithiodinicotinic acid, this being sufficient to provide the system with a 10^{-3}M concentration of said acid. A portion of the resulting acid-treated mixture was then used as such in the manometric tests. The remainder of the acid-treated cells were then separated by centrifugation washed twice with the buffer and then resuspended in the same buffer to form a

stock suspension containing approximately 150 x 10^6 cell/ml. which was then used for manometric tests. In said tests, the experiments were run for one hour at 37°C. using the method described in detail in the article, "The Effect of Some Sulfur-Containing Pyridine Derivatives on the Carbohydrate Metabolism of Ehrlich Ascites Tumor", Grassetti et al, Journal of Medicinal Chemistry, 8,753 (1965), except that in the present tests no further heparin was employed after the initial cell-washing steps, as described above. The results obtained are expressed below in Table 2 wherein:

Q_{O_2} = oxygen uptake in μl O_2/hr/mg dry weight. The gas phase was air, the dry weight 26.6 mg/flask, the number of cells 60 x 10^6.

$Q_{O_2}(G)$ = same as for Q_{O_2} except for the addition of glucose added to provide a 0.05M concentration.

$Q_{CO_2}^{O_2}$ = aerobic CO_2 evolution in μl CO_2/hr/mg dry weight. The gas phase was O_2-CO_2 (95:5). The dry weight, cell number and glucose concentration were the same as for Q_{O_2} (G).

$Q_{CO_2}^{N_2}$ = anaerobic CO_2 evolution in μl CO_2/hr/mg dry weight. The gas phase was N_2-CO_2 (95:5), the dry weight 13.3 mg/flask, the number of cells 30 x 10^{16}/flask and the glucose concentration 0.01M.

In each case the total liquid volume per flask was 3.0 ml.

Table 2

EFFECT OF TREATMENT WITH 6,6'-DITHIODINICOTINIC ACID ON RESPIRATION AND GLYCOLYSIS OF EHRLICH ASCITES TUMOR CELLS

	Q_{O_2}	$Q_{O_2}(G)$	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N_2}$
Ehrlich ascites cells (control)	-4.9	-3.7	+ 11.8	+ 28.1
Ehrlich ascites cells, in presence of 10^{-3}M CPDS	-4.8	-3.3	+ 14.6	+ 30.7
Ehrlich ascites cells, treated 5 min. with 10^{-3}M CPDS, then washed	-4.7	-3.6	+ 11.9	+ 28.9

The data of the above Table 2 show that treatment of Ehrlich Ascites cells with 6,6'-dithiodinicotinic acid at a concentration of 10^{-3}M, followed by washing, does not alter their rates of respiration and glycolysis. Respiration and glycolysis of Ehrlich ascites cells in the presence of 10^{-3} 6,6'-dithiodinicotinic acid are also essentially unaffected.

When the foregoing operation is repeated using 2,2'-dithiodipyridine as the reagent, evolution of CO_2 and O_2 substantially ceases after several minutes, thus indicating that this reagent is strongly inhibitory of cell glycolysis and respiration and thus is cell damaging.

Example 3

a) This operation was conducted to determine the degree of presistence of the cell-blocking action in the living mammal. Swiss mice which had been inoculated with ascites cells approximately 6 days before the test, were each injected intraperitoneally with an aqueous solution containing 6 mg. of 6,6'-dithiodinicotinic acid sodium salt, this dosage being equivalent to 300 mg. of the reagent per kilo of body weight. This salt had been prepared by adding the acid to an aqueous solution of sodium bicarbonate present in the amount stoichiometrically required to neutralize the acid and thus solublize the same in the aqueous solution. The latter was then brought to a pH of 7.2 by addition of KRP buffer, as required, before being injected into each mouse. As indicated in Table 3 below, a certain number of mice were sacrificed at intervals of 1, 4, 8 and 20 hours after the reagent was injected. After the indicated time, the ascites fluid was withdrawn from the animal and centrifuged to separate the cells which were then washed 5 times with KRP buffer. Each lot of cells so recovered (the number of which in each mouse averaged from 1 - 2 x 10^9) was treated with a solution of 5 μ mole of cysteine. The resulting product was centrifuged (5 minutes at 800 x g) following which the supernatant was denatured for 5 minutes at 95°C and then analyzed for thione by observation of the peak at 344 mμ. The value observed at the end of the first hour was arbitrarily taken as 100, and is so expressed in Table 3 below. It will be seen that cell blockage substantially ceased between the 4th and 8th hours.

Table 3

RATE OF RELEASE OF BLOCKED -SH GROUPS

ABOUT ASCITES CELLS IN LIVING MICE

No. of Mice	Hrs. Following Treatment	Blocked Cells Remaining %	No. of Blocked -SH Groups per Ascites Cell *
5	1 Hr.	100	1.7×10^8
4	4	33	5.5×10^7
6	8	1	1.9×10^6
4	20	0	0.00

* It will be noted that each -SH group, which has a 0 electric charge at pH 7.2 ± 0.2, is replaced by a carboxy group which bears a negative charge at said physiological pH level.

b) The mice forming the subject of the above experiment showed no signs of discomfort when injected with the acid salts. However, in a companion test conducted in the same manner, but using a suspension of the free 6,6!-dithiodinicotinic acid in a 3% gum arabic solution in KRP buffer at pH 7.2, the (5) mice employed in the test exhibited drowsiness for several hours. Further, at the end of 4 hours after treatment, it was found that 65 percent of the peripheral -SH about the ascites cells still remained blocked. This is attributable to the fact that the injected reagent, being insoluble, was taken up at a slow rate and thus was able to effect a continuing reaction with the peripheral cell groups as it was slowly converted in vivo to the soluble salt form.

c) In still another test run as a control, the mice were injected with a saline susp. of 6,6'-dithiodinicotinic acid, and here again the symptoms of drowsiness were observed, thus showing that the gum arabic employed above was not a causative factor.

Example 4

Each of 6 healthy mice, free of any tumor or other disease, were injected intraperitoneally with 18 mg per day of 6,6'-dithiodinicotinic acid sodium salt for 21 consecutive days, equivalent to a daily dosage of 450 mg per kilo of bodyweight. The injected chemical was in the aqueous, pH 7.2 form, as

described above in Example 3(a). The mice exhibited no visible signs of drowsiness or other ill effect during the period of the test or thereafter. Their appetites remained good and no weight loss was suffered.

Example 5

Ehrlich ascites tumor cells were withdrawn from one or more 6-7 day ascites mice, the cells counted and diluted with physiological saline to an appropriate concentration. Concentrations between about 100,000 and 500,000 cells per 0.01 ml were used. The preferred concentration was about 200,000 cells/0.01 ml. Cell suspension (200,000 cells, 0.01 ml) was injected into the brain of 48 mice, using a Hamilton syringe and 25 gauge needle (5/8" long). Part of the needle was covered by polyethylene tubing (0.018 I.D.), so as to limit the penetration of the needle into the brain to 6 mm. The needle was inserted into the midbrain through the squamopetrosal fissure.

The mice were divided into two groups of 24 mice each. Those of group A received an injection IP of 2 mg 6,6'-dithiodinicotinic acid in sodium bicarbonate solution (0.1 ml) every 4 hours thereafter, starting within not less than one hour and not more than 3 hours after the intracerebral injection, and for 7 consecutive days and nights, or 42 injections. Each mouse of the second group B, received an injection of physiological saline solution (0.1 ml, IP) every 4 hours, given at the same time as the injections of group A, for 7 consecutive days and nights, or 42 injections.

At the end of 7 days, the mice were sacrificed and their lungs excised and minced. Two portions of about 80 milligrams of minced lung tissue from each mouse were implanted subcutaneously in the retroscapular area of a healthy mouse, on both sides. We thus had 24 mice containing 48 implants of lungs of group A mice, and 24 mice containing 48 implants of lungs of group B mice.

These mice were kept without any special treatment for 30 days, then sacrificed and any tumors that had formed at the site of the lung inplants were excised and weighed. A tumor weighing 100 mg. or more was considered a "take" i.e. the lung tissue from which it originated contained a metastasis.

The results obtained in this experiments are given below:

	No. of Takes / No. of Implants	% Takes	Average weight (1) of Tumors
Group B (not treated)	24/36	67	179 mg
Group A (treated)	8/46	17	59

(1) Average total weight of all tumors, including those weighing less than or more than 100 mg.

These results show that 6,6'-dithiodinicotinic acid, when administered as indicated, has the effect of decreasing the number of takes to 25% that of untreated mice, and of decreasing the total tumor mass to 33% that of untreated mice. It is known that still higher doses of the 6,6'-dithiodinicotinic acid reagent, which may lead to further improvement, are well tolerated.

Example 6

The experiment of example 5 was repeated, and the following results were obtained:

	No. of Takes / No. of Implants	% Takes	Average Weight of Tumors
Group B (not treated)	28/44	64	547
Group A (treated)	7/46	15	177

Example 7

	No. of Takes / No. of Implants	% Takes	Average Weight of Tumors
Group B (not treated)	14/48	29	133 mg.
Group A (treated)	3/44	7	79 mg.

The dosage rate of the dicyclic or other reagent to be employed in a practice of this invention can be varied within wide limits and still be effective for the intended purpose. In general, however, good results can be obtained by administering the reagent in amounts calculated to maintain a concentration in blood of 10^{-6} to 10^{-3} molar. So employed, the non-toxic, preferentially peripheral -SH bonding reagents have utility in modifying and moderating various undesirable inter-cellular reactions involving the cell periphery such as those encountered in tumor metastasis and invasiveness. For example, an advantageous use of the 6,6'-dithiodinicotinic acid herein described

consists in the administration of adequate doses at the time of surgery for cancer. It is known that manipulation of a tumor during surgery causes numbers of cancer cells to enter the blood stream with concomitant high danger of formation of metastases. Treatment with the reagent should be continued for an adequate period of time both before and following surgery until the natural defenses of the organism have destroyed the remaining circulating cancer cells.

The dicyclic or other reagent employed in a practice of this invention to react preferentially with peripheral -SH groups of cells can be administered to living mammals in any desired fashion such, for example, as by injection into the blood stream or into muscle or body tissue. Oral administration may also be effective. The chemical can be employed per se or along with an oleaginous or other vehicle which will slow down its rate of absorption into the system. It is also possible to use a reagent of the foregoing character which is prepared in radioactive form by incorporating one or more radio-active carbon, sulfur or tritium atoms. By use of this practice the efficiency and efficacy of the radioactive elements are greatly enhanced inasmuch as they become chemically bonded to the cells for the desired interval.

SUBSTITUTE

REMPLACEMENT

SECTION is not Present

Cette Section est Absente